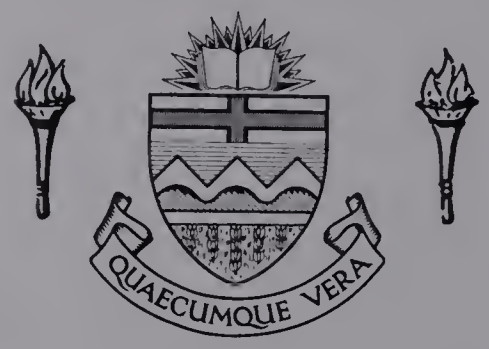

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A STUDY OF METHODS FOR DETERMINING THE VIABILITY OF A CORNEA

by

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A THESIS

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The undersigned hereby certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "A STUDY OF METHODS FOR DETERMINING THE VIABILITY OF A CORNEA", submitted by Ronald G. Jans, M.D. in partial fulfillment of the requirements for the Degree of Master of Science (Surgery)

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ABSTRACT

Information gained from studies over the past twenty years has provided the basis for the development of a number of tests of tissue viability. Two of the principles derived from these studies are of interest to this research work. The fact that corneal metabolism and subsequently corneal thickness vary significantly with temperature change and the observation that cellular viability has been accurately determined with the use of a supravital stain (Lissamine green) are those principles.

In this study the comparisons made between the above principles were accomplished using corneas from the common house cat. Each cornea was measured for thickness immediately following enucleation, after refrigeration at 4°C and after incubation at 37°C. The percentage decrease in thickness occurring after incubation was then calculated. Each cornea was also observed after the incubation period for the presence of dead endothelial cells as indicated by staining with Lissamine green. Some corneas were stained immediately after enucleation so had no other procedures performed upon them. The mean number of cells stained per photograph was then tabulated.

A statistical study was then undertaken in order to discover if a correlation exists between cellular death, percentage decrease in thickness and increasing time in storage following enucleation. Within the limits of this study the following conclusions can be expressed:

1. There is a significant correlation between the number of dead endothelial cells shown by a positive staining reaction and the percentage decrease in thickness at given time intervals in

storage after enucleation.

2. Determination of corneal endothelial viability with this supravital staining technique at this time and in our hands is a more accurate and more practical method than that using the Temperature-reversal effect per se.
3. The supravital staining technique is a simpler, more sensitive indicator of endothelial cell viability than is described in the present literature.
4. There is a significant relationship between the number of dead endothelial cells and the increasing time in storage following enucleation.
5. There is a significant relationship between the percentage decrease in thickness and the increasing time in storage following enucleation.
6. More work should be done to test the feasibility of these methods in a clinical situation.

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I. INTRODUCTION

For many years at various institutions in which keratoplasties are undertaken, there has been a need for a simple, accurate and practical method to determine the viability of a cornea. The wide variations in the appearance and condition of corneas received in eye banks due to factors including age, variable refrigeration time, tissue damage and individual idiosyncracies are all important considerations in determining this need. Although there are many methods available to indicate corneal viability, there appear to be few that will indicate the viability of the cornea without rendering the tissue unsuitable for a future graft.

The aim of this research was to improve upon the methods now being used to select donor material for keratoplasty by developing a new technique to indicate the state of viability of the corneal endothelium. We hoped that by improving the selection procedure for donor material there would be an increase in success rate of future transplants.

The examination of a cornea from an enucleated eye using a slit lamp only, will indicate that that cornea appears morphologically identical to one present in a living animal. However, because the cellular activity is not indicated by this method, its viability is also unknown. A number of investigators have now shown that the metabolic integrity of the epithelium (Harris and Nordquist[32], Harris[33]), endothelium (Davson[13,14], Langham and Taylor[55], Aurell, Dohlman and Roden[2]) and even the stromal keratocyte (Langham[56]) is important in the hydration control of an in vivo or in vitro cornea.

The Temperature-reversal effect was first described by Davson in 1949. This "effect" is the phenomenon of expansion (swelling) of a

cornea in a moist atmosphere as the temperature is reduced to 4°C and the return to or near to normal thickness as the temperature is brought back to body temperature or slightly higher. This has been shown to be related mainly to the function of the corneal endothelial layer and therefore as such could possibly be thought of as an indicator of the viability of the endothelium.

Along with the use of the physical tests for viability a number of stains, especially so-called supravital stains, have been advocated for use in determining viability. Of these, we felt that Lissamine green was the best choice for our present purposes. Holmberg[42] had reported results of his work and had found it a simple, accurate and direct method of determining a cell's viability while in suspension. Each cell upon suffering irreversible damage immediately became stained by the Lissamine green but not before.

With the evidence reported by the previously mentioned investigators at our disposal we were influenced to investigate the possibility that one of these methods might be valuable in corneal viability studies.

The object of our research then, was to compare the Temperature-reversal effect with a technique involving Lissamine green staining as indicators of corneal viability.

The Temperature-reversal effect was studied by measuring corneal thickness with the pachymeter immediately following enucleation, after refrigeration and after incubation. Photographs of the endothelial surface stained by Lissamine green were taken following incubation and the mean number of stained cells per photograph was calculated. Only the mean percentage decrease in thickness after incubation was statistically

important in the Temperature-reversal portion of this experiment as it appears to be closely related to the functional metabolic integrity of the cornea.

Our initial intent was to determine the efficacy of the Temperature-reversal effect as an indicator of corneal viability so a statistical analysis was finally initiated to find if a relationship was present between it and the staining method of determining viability.

A. STATEMENT OF THE PROBLEM

The main problem contained within this study is to establish a relationship between the Temperature-reversal effect (mean percentage decrease in corneal thickness after incubation) and the Lissamine green staining procedure (mean counts of dead endothelial cells) as to their abilities to indicate corneal viability.

B. SUBSIDIARY PROBLEMS

1. To establish a simple staining technique (using Lissamine green upon a wholemounted specimen) for the corneal endothelium.
2. To establish a precedent so that a subsequent piece of research might attempt to apply our results to an operative trial.

II. REVIEW OF THE LITERATURE

The advent in the past several years of various tissue transplantation techniques has promoted a great deal of interest in preservation and viability studies of the donor tissues involved. This may be said to be of particular import in the field of Ophthalmological research in that donor corneas used for penetrating keratoplasty are often difficult to assess as to their probability for success.

The technique of vital staining probably initiated the search for methods to accurately determine tissue viability. Vital (Intravitam) staining is said to date from the work of Bouffard in 1909 and Goldmann in 1909. Bouffard's work consisted mainly of the use of basic dyes and in some cases suspensoid preparations whereas Goldmann used some acid colloidal dyes as well. According to Cappell (1929) the first real breakthrough in the technique of supravital staining came with the works of Michaelis (1899), Bensley (1911), and Cowdry (1918). These men were apparently the first to recognize the vital staining of the cell's mitochondria. The supravital stains in use at that time were mainly basic in nature with very small molecular structures.

The first work done in this particular field that was related to Ophthalmology was that produced by Matsumoto in 1918 who, with the aid of the vital dye Neutral red, stained the corneal epithelium and produced the statement that in healthy cells the nucleus never became stained by a supravital technique. In 1929 Cappell published a large review of developments produced by vital and supravital staining techniques in relation to the study of cellular viability. Although there was no Ophthalmic import in this treatise, a description of other body tissues

TABLE I.

CORNEAL VIABILITY STUDIES

1) Appearance of donor material on examination with slit lamp.
2) Histological appearance of donor material.
3) Special histological features e.g. acidophilia.
4) Loosening of epithelium.
5) Cell migration and mitotic activity.
6) Respiratory activity.
7) Thickness of the cornea and water uptake.
8) Growth in tissue culture.
9) Supravital and vital staining.
10) Phase contrast microscopy.
11) Mineral components analysis and permeability to ions.
12) Nitrogen fractionation.
13) Activity of proteases.
14) Reduction and Oxydation carriers.
15) Carbohydrate fractionation.
16) Lipid fractionation.

stained with various vital and supravital dyes was produced in great detail.

The interest in supravital and vital staining techniques resulted in the development of a number of stains for use in tissue viability determination experiments. At the same time, however, there appeared a desire to find some other method that would be more accurate in measuring the viability of a tissue. In the past number of years then, many methods of determining tissue viability have been described.

Corneal viability studies have produced a number of "viability tests" as well, (Table I.). A number of these methods, however, encounter problems of interpretation and technique.

The appearance of the donor corneas on slit lamp examination and the phenomenon of loosening of the epithelium were the earliest methods used to determine the viability of the donor tissue. These methods, however, are rather crude and subject to clinical error. Various other methods (e.g. the development of special histological features and the production of cellular migration and mitotic activity in the epithelium) (Bushke, 1951) produced results little better than the prior clinical methods.

(a) Respiratory Activity

In 1948 Duane produced evidence using the Warberg apparatus that the metabolic activity of the cornea and hydration, or at least transparency, were probably related. He hypothesized that the respiratory activity of the cornea, if measured, might give information regarding the cornea's viability. However, he found that the functional integrity of respiratory enzymes in cells is prolonged beyond time periods in which a corneal graft

had been found to be successful.

Following that report Langham, in 1951 and 1952, stated that a decreasing oxygen content produced a hazy cornea as well as a marked rise in the lactic acid content of the cornea. These findings indicated that an aerobic metabolic process was important in the maintenance of a viable cornea. Aurell, Dohlman, and Roden in 1956 then reported that procedures using the Warberg apparatus and also S^{35} uptake studies confirmed findings that Duane reported in 1948. They also found that the moist chamber method and the liquid paraffin method of storing donor corneas sustained viability for longer periods than a number of other storage conditions that had been advocated.

(b) Corneal Thickness and Water Uptake

According to Bushke (1951) the study of the relationship between water imbibition and the change in corneal thickness began with Papenko in 1936 although this is not well recognized. Cogan and Kinsey in 1942 produced a number of conclusions that greatly influenced the investigators of the day. Their research considered the cellular layers of the cornea as semi-permeable membranes that were exposed anteriorly and posteriorly to hypertonic solutions (tears and aqueous humor respectively). The state of hydration of the cornea was arrived at via the continuous osmotic abstraction of water from anterior and posterior surfaces and an equilibrium was established between osmotic forces at the corneal surfaces and the imbibitory power of the stroma. This proposed theory of course implied the relative lack of electrolyte movement through the "semi-permeable membranes" (epithelium and endothelium) of the cornea.

Davson in 1949, however, stated that salts as well as water were taken up by the cornea upon storage in a cool atmosphere and postulated that it was aqueous humor rather than just the water that was moved. He also found and reported the first article on a "Temperature-reversal effect" in that ox corneas tend to swell when cooled and to return to normal when warmed. This was followed in 1951 by Maurice's paper describing his evidence that the endothelium and epithelium were indeed permeable to salts (at least to sodium) and that osmotic diffusion if at all was not the only mechanism utilized in dehydrating the cornea.

Following this many investigators began producing evidence that not only refuted Cogan and Kinsey's initial theory but also supported the growing opinion that the metabolic integrity of the cellular layers was most important in sustaining a cornea suitable for grafting. In 1954 Schwartz, Danes and Leinfelder showed that the hydration of the cornea was influenced not only by temperature and anoxia but also by the glucose content of the metabolically active cells, pH, and the presence or absence of aerobic enzymes. Their conclusion was that the maintenance of a constant water content in the cornea consumed a great deal of energy and that this energy was mainly required to prevent the hydration of the tissue. Subsequent studies by many investigators (Davson 1954 and 1955, Harris 1957, Langham 1956, 1960 and 1962, and Maurice 1962) have confirmed the initial theory that metabolism has an obligatory role in the maintenance of corneal hydration and also that an active transport system must exist somewhere within the corneal tissue. In 1956 in a detailed investigation Langham and Taylor showed that as temperature decreased corneal thickness increased and that as oxygen supply

to the cornea decreased a mild increase in thickness occurred initially but after three hours this became quite rapid and marked. They concluded that aerobic and not anaerobic metabolism was necessary for the maintenance of normal corneal hydration.

Lavergne in 1963 and Hassard in 1964 both reported experiences with their use of the Temperature-reversal effect as an indicator of corneal viability. They found, independently, that corneas when cooled at 4°C. for 12 or 24 hours and then incubated at 37°C. with 95% oxygen never did return fully to normal thickness. Also reported was their finding that the endothelial mosaic never did return to its normal appearance and that the longer the eye was stored at 4°C. before incubation the less striking was the reduction of corneal edema. Both Hassard and Lavergne found that the Temperature-reversal effect was still present even after six days of refrigeration at 4°C. It was therefore shown that the Temperature-reversal effect could be demonstrated long after a cornea would normally be discarded as donor material by other criteria.

The consensus then, appeared to be that a metabolic process of active transport was responsible for the hydration control of the cornea and that the lack of this process prevented the subject cornea from behaving as it normally should in a living state.

The close relationship of the state of hydration and corneal thickness demanded a reliable method of measuring thickness in both in vivo and in vitro corneas by optical means. According to Donaldson (1966), Blix in 1879 - 1880 produced the first instrumentation for this purpose. He was followed by Gullstrand in 1924 who attempted through a series of rather complicated procedures to measure thickness with a theodolite.

In 1921, Hartinger, the first to use the slit lamp for this purpose devised a method that produced at first only apparent readings that were then corrected to give the actual value. Juillerat and Koby in 1928 used the same principle only modified it by the use of an eyepiece micrometer. Goldmann (1932) used the first split ocular to measure the optical section of the cornea produced by the slit lamp beam.

Von Bahr in 1948 in a number of studies based on the technique of Blix produced accurate measurements with his method, however, disadvantages listed by Maurice and Giardini (1951) and the development of their simpler method led to the adoption of their optical technique for measuring thickness accurately (pachymeter). A modification of this technique by Donaldson in 1966 produced an accurate optical method of measuring corneal thickness using Maurice and Giardini's principle but with the addition of the split ocular. This modification allows superimposition of the optical section's halves accurately upon one another. The development of the pachymeter produced measurements with an error of only $\pm$ .1 mm. and the establishment of the presence of an optical zone approximately 3 mm. in diameter near the center of the cornea that produced measurements of thickness accurate to $\pm$ .01 mm.

(c) Growth In Tissue Culture

In years past tissue culture techniques for determination of corneal viability have been widely investigated. Kobzar (1935), Bajenova and Filatov (1936-1938) were the initiators of this particular procedure in relation to the cornea. Since then many workers including Stocker (1958, 1963), McPherson (1956), Smith, Ashwood-Smith, and Young (1963), and DeOcampo (1965) have developed more refined techniques for determining

the viability of cell layers of the cornea by various tissue culture techniques. These workers, expecially Stocker, have influenced surgeons performing keratoplasties to adopt the 48 hour time limit after death of the donor subject as the time after which success rates declined rapidly. This time limit has been supported by other investigators with different procedures since that time.

(d) Supravital and Vital Staining

Meanwhile, as the activity in metabolic and tissue culture studies of the cornea continued, there was a renewal of interest in the development of both old and new vital and supravital staining techniques - this time in relation to the cornea's viability. Hanks and Wallace in 1958 supported the use of Eosin-Y (DE-5) and stated that due to the materials, time and the attention required to assess viability by gross determination (metabolic and tissue culture techniques) it would be useful to emphasize more the use of dye exclusion theories and techniques. In other words to use stains that are denied incorporation within a cell because of its viability state.

In 1961 Holmberg produced a very significant paper discussing the permeability of a number of vital stains in the course of a cell's injury and then death. His comparisons were made using Eosin-Y, Erythrosin B, Lissamine green, Methylene blue, Nitro-blue tetrazolium, Nigrosin, and Trypan blue, and were based upon the observations of a number of other investigators beside himself with these particular dyes. The results of his study clearly indicate that of the above mentioned vital stains only Methylene blue and Trypan blue will penetrate a living cell's membrane. The long-term exposure of living cells to Lissamine green, however, led

to the pinocytotic incorporation of small amounts of the dye into the cells. This picture under the microscope was much different than that observed when an irreversibly damaged cell was exposed to the stain. In these cases the dye instantaneously penetrated into the cell, staining the denatured proteins of the cytoplasm and nucleus caused by the fatal injury. Under these circumstances consistent and reproducible differential counts of damaged cells were obtained with Lissamine Green and Trypan blue. With Nigrosin and Erythrosin B, however, higher counts were generally obtained and with Eosin-Y an interpretive difficulty was encountered that allowed a very high observer error. The claim that Nitro-blue tetrazolium was reduced by undamaged cells was substantiated by Holmberg at least in part but he also found that cells permeable to Lissamine green showed the reduced Diformazan crystal in the cell in quite large quantities. He, therefore, doubted that the tetrazolium reaction was a suitable means of determining the viability of cells.

Holmberg's data on Lissamine green indicated that the cells exposed to the dye became freely permeable first when metabolic activities ceased as indicated by the lack of membrane activity. His work impressed upon us the point that a permeability test for cell vitality that binds a chemical to the denatured protein of a cell incurred during an injury is much more accurate than an enzyme reaction that tended to overlap from an undamaged to an irreversibly damaged cell.

In 1964, however, two groups of investigators reported findings as regards corneal viability using the Nitro-blue tetrazolium technique of staining the endothelium. Pẽna-Carrillo and Polack found that as cold storage time in a moist atmosphere increased, the concentration of the Diformazan crystal in the cell increased and the number of cells that

became involved increased. This then implied that the Nitro-blue tetrazolium was an indicator of the death of a cell.

Kaufman, Cappella, and Robbins first in 1964 and since then with a number of articles have proposed the use of Nitro-blue tetrazolium again, as has Stocker, only in a different way. Their methods involved the use of a freezing technique (-75 to 80°C for 30 minutes) first to make the cells permeable, then incubation with the stain after the desired procedures had been performed. Their staining results supported their contentions that as cell death occurred the oxydative enzymes in the cell were lost and that staining would not occur. However, as Holmberg previously stated there was no delineation between cells reversibly damaged and those irreversibly damaged with their method. This indication plus the fact that the measurements of media and complication of incubation with the material in the stain produced some resistance, at least in our laboratory, to the use of it as a viability indicating stain.

Preziosi (1966) has now reported good results with phase microscopy in observing changes in cells during freezing and thawing procedures. In his examples morphological changes are easily recognized with this particular method. As well as his observations on the efficacy of phase contrast microscopy in differentiating living from dead cells in the corneal endothelium he also suggested the presence of some sort of substance coating the endothelium that appears to lose its binding properties when cooled and though the cells are intact this substance appears as some type of debris covering the cell layer. No further comment has appeared in the recent literature about his observation.

The universal acceptance of a viability test for the cornea is still

not reality. The sooner this occurs the quicker more meaningful experimentation in the realm of corneal transplantation will be accomplished.

III. THE ANATOMY AND PHYSIOLOGY OF THE CORNEA

A. ANATOMY [1,15]

The cornea is a curved, transparent, avascular, highly organized collagenous tissue, bounded at its anterior and posterior surfaces by cellular membranes and itself bounding the anterior chamber of the eye. The radius of curvature of the central cornea is approximately 7.86 mm. whereas towards the periphery it becomes less curved. An area approximately 3 mm. in diameter at or near the centre of the cornea (the optical zone) has been found to be fairly constant in thickness at .50 to .55 mm. and also to have a very regular curvature. When viewed from the front the cornea appears to have an oval shape (greatest diameter horizontally) but from behind it is circular with a diameter of about 11 to 12 mm. The total superficial area of the corneal surface is 1.3 $cm.^2$

The cornea has five anatomically distinct and separate layers although one more may be included (tear film) as its function is related to corneal integrity. These layers are as follows:

1) Tear Film: This film lies anterior to the epithelium and provides an interface that acts as a refracting surface between the cornea and the air. The film is derived from secretions of the lacrimal, Meibomian, and unicellular mucous glands. It appears to have two separate layers; an oily layer from the Meibomian gland anteriorly that prevents evaporation from the corneal surface, and an aqueous layer that coats the epithelial surface with an isotonic (not hypertonic) solution.
2) Epithelium: A cellular stratum that is composed of 5 - 6 cell layers and makes up about 10% of the total corneal thickness. The basal

cell layer consists of columnar cells and the middle cell layers contain wing cells that become wider, thinner and flatter as one proceeds superficially. The superficial cell layer consists of flat squamous cells that normally never become keratinized and appear to have many microvilli projecting into the aqueous layer of the tear film. In the basal cell layer another type of cell, "the Langerhans cell", has been discovered but its function is not yet known. The corneal epithelium has been estimated to be completely renewed once every week. The basement membrane consists of a number of minute fibrils imbedded in a homogenous matrix rich in glycoprotein and phospholipids. It varies in thickness from 200 to 300 Å and attaches to basal cells in a number of places as well as sending fine filaments into the stroma.

3) Bowman's Membrane: A transparent almost structureless sheet of tissue (with light microscope) that in actual fact consists of very fine collagenous fibrils running parallel to one another. It is about 12 microns thick.

4) Stroma: A structure contributing to 90% of the cornea's total thickness and composed of regular lamellae of collagenous fibres lying parallel to its surface. Each lamella extends from limbus to limbus and is about 2 microns thick. The fibres of alternate layers appear to run at nearly right angles to each other with the orientation becoming less exact as the limbus is approached. The fibres lie in a ground substance of protein, glycoprotein and mucopolysaccharides.

There are two types of stromal cells present; the majority being fixed cells or keratocytes and the others that are wandering cells or

histiocytes that are distorted in shape by the lamellar pressure.

5) Descemet's Membrane: Under the light microscope a homogenous mass bounding the stroma's posterior surface and about 10 microns thick. The electron microscope, however, shows a meshwork of a large number of collagenous sheets of an atypical form. It is elastic but its chemical nature and structure is not like that of elastin but rather of collagen. It is highly resistant to digestion by enzymes or alkalies but is permeable to solutes of various sizes.

6) Endothelium: A single cell layer about 5 microns thick that because of a mesodermal origin should probably be called a mesothelium rather than an endothelium. The cells are polygonal in shape, regular in size and appear to form a mosaic over the posterior corneal surface. The cells rest on Descemet's membrane (rather like a basement membrane for them) and appear to be responsible for its presence. The cytoplasm is abundantly supplied with organelles especially mitochondria except in the most superficial portion adjacent to the next cell where a terminal web of very fine interwoven fibrils is seen to join the juxtaposed cells. This terminal web appears to arrest fluid or solute transport through the intercellular space. The endothelial cells much unlike the epithelium form a very stable population that is replaced only if an injury of some form activates a mitotic replacement of cells.

B. CORNEAL PHYSIOLOGY [1,15] - Relative to Hydration-Dehydration Properties

The cornea has four properties or characteristics that in some way play a large part in its hydration-dehydration functions. These properties may be dealt with as follows:

1) Stroma: a) Swelling pressure and Imbibition pressure

b) Transparency

2) Cellular Layers: a) Permeability

b) Control of Corneal Thickness

Although these particular entities are separated for purposes of discussion it must be realized that each is unalterably a function of the others.

1) Stroma:

a) Imbibition Pressure: This pressure may be defined as the force tending to produce swelling in the cornea. A number of other forces exert themselves upon the internal and external surfaces of the cornea and will be defined now for future reference. The swelling pressure is the pressure that is required in order that the cornea will be prevented from swelling. The tissue pressure is the expansive force created by the structural elements of the stromal framework and any other ground substance components so composed as to exert a steady force upon individual fibrils. The fluid pressure is merely the hydrostatic pressure that fluids possess under normal circumstances, however, the osmotic pressure, the force tending to draw water into the stroma produced by solutes present in the tissue framework, can influence it as well.

As is generally understood, an isolated section of corneal stroma if exposed to a moist atmosphere will swell due to the imbibition of fluid. The swelling occurs in a direction perpendicular to the corneal surface and in the intact eye it appears to be related to the integrity of the cellular layers of the cornea whether in vitro or in vivo. Also as the swelling

increases the swelling pressure within the stroma appears to decrease.

It is now generally believed by most authorities (Dohlman and Anseth,[18] Hedbys and Dohlman,[40] Hedbys,[37] Mishima[67] and Maurice[65]) that whether in vivo or in vitro the cornea is being subjected to a constant force attempting to make it swell or expand. These investigators have generally agreed that the energy required to maintain this force is supplied mainly by the mucopolysaccharide portion of the stroma the medium that is now implicated as the site at which the swelling occurs. This has been supported by electromicrographic evidence that there is no change in the appearance of the stromal collagen as the swelling takes place (Francois, et al 1954).

In 1963 Hedbys, Mishima and Maurice produced an excellent monograph that clearly described the relationships of the various "pressures" previously defined. According to their work in which various pressures were intimately related they found a number of interesting relationships. They were originally intending to measure the imbibition pressure and in so doing determined other pressure relationships within the cornea. The finding of prime importance was that the imbibition pressure (IP) in a cornea lying free in saline was equal to the swelling pressure (SP) with a negative sign. In the cornea of an intact eye the relationship was that the imbibition pressure was equal to the intraocular pressure, less the swelling pressure. That is, a negative value in most normal circumstances. The pressure

relationships are very difficult to understand unless viewed under an algebraic formulation as put forward by Hedbys, Mishima and Maurice in their monograph.[39]

The swelling pressure has been calculated as being 70 - 80 mm Hg. and the normal intraocular pressure around 15 mm. Hg. Applying these figures to the previous relationship one is left with an imbibition pressure of -55 - 65 mm. Hg. which is the value obtained by Hedbys, Mishima and Maurice.[39]

Langham,[56] on the other hand, suggested that rather than a swelling pressure being normally present in the stroma, possibly the pressure was usually zero due to a balance between the imbibition pressure and the cohesive forces of the cornea's structural elements. According to this theory swelling would result when the cohesive forces were reduced. This would produce a concurrent decrease in imbibition pressure and a new equilibrium would then be established. This particular view, however, has not been widely substantiated by other investigators.

b) Transparency: Transparency may be defined as the property of a structure that allows the transmission of light rays to enable objects to be seen through that structure.

The cornea transmits nearly 100% of light rays in the visible spectrum and therefore is considered a transparent structure. Until recently it was generally assumed that corneal transparency resulted from the uniformity of refractive index of its components. However, more refined procedures have now shown that the refractive indices of the collagen and ground substance are quite different (1.550 and 1.345 respectively). Therefore, the initial

theory has since been abandoned in favour of Maurice's (1957) theory based upon the refractive index and other physical properties of the cornea.

The phenomenon of stromal transparency can only be explained in terms of the optical properties (the size, shape and refractive index) of its components. It has also now been shown that the structural unit, the lamella, does not appear to be the basic factor.[65] The absence therefore of resolvable structures responsible for corneal clouding and the greater scattering of light of shorter wavelengths indicates that the components responsible are of submicroscopic dimension. Electron microscope studies indicate that the corneal lamellae consist of collagen fibres made up of many submicroscopic fibrils lying parallel to one another. The properties of these structures involved in transparency are the diameter of the fibrils and the refractive indices of both the surrounding medium and fibrils.

The fibrils (dia. 250 Å) are believed to be arranged in a regular two dimensional lattice structure with each fibril equidistant from its neighbours. Because the spacing of the fibrils is less than the wavelength of visible light there appears to be a mutual interference to the scattered light rays. There is, therefore, essentially no light scattered except in the direction of the incident beam and this according to the theory constitutes a transparent structure. According to Maurice (1962) evidence to support the lattice structure theory has been produced using x-ray diffraction techniques as well.

Maurice also suggests that a direct repulsive force between fibrils could possibly be mediated by a substance coating the fibrils, probably the mucoid involved in the ground substance contents.

2) The Limiting Cellular Layers:

a) Permeability:[1,15] Permeability of the cornea was first explained by a "Pore Theory" that regarded the tissue as a sieve-like membrane through which substances passed only on the basis of their molecular or ionic size. Some substances of large molecular size, however, were found to penetrate the cornea while some of quite small size were unable to do so. In view of this fact a second hypothesis based on the electrostatic qualities of the cornea was proposed. This argument which considered passage through the cornea relative to the presence or absence of an electric charge, however, could not explain the permeability of many non-polar compounds that existed.

A more recent theory of permeability that is based on the solubilities of substances has more recently been advocated and has been accepted much more readily than the others. Accordingly the cornea is considered as a membrane consisting of a number of different solvents through which substances may pass if they possess the appropriate solubility characteristics.

When considering permeability of non-ionic compounds fat soluble substances have been found to be more soluble in the epithelial layers probably due to the presence of a high lipoid concentration in this layer. Substances highly soluble in

aqueous solutions have been shown to pass easily through the stromal layers but not through the epithelial layer. It has also been shown that substances soluble both in aqueous and oily solutions pass through the entire cornea with little difficulty. The permeability of the cornea therefore appears to be related to the property of phase solubility, the epithelium being permeable to substances with a fat solubility phase and the stroma to substances with a water soluble phase.

Other properties much as the state of dissociation, the tonicity and the polarity of a substance are influencing factors upon the solubility of various substances. It has been shown that the cornea becomes more permeable to substances as they become more associated. That is, as weak bases progressively loose charges (pH going up) and weak acids gain charges (pH going down) the permeability to these substances increases. Polarity is important in that non-polar compounds are more fat soluble than polar compounds and therefore penetrate the epithelium more readily. It is interesting to note, however, that if these compounds are carried in vehicles unlike themselves (polar compounds in non-polar vehicles, etc.) the total corneal permeability increases. When considering ionic substances the permeability across the intact cornea increases when the pH of the solution falls outside of the range 4 - 10. The greatest resistance to penetration of these substances is in the epithelial layer for when it is removed or severely damaged, ionic solutions will easily pass through the stroma. Various investigators have also

shown that the passage of ionic material through the intact cornea is influenced by conditions that affect corneal metabolism. The theory put forward is that active transport is an important factor in the passage of these substances through the corneal layers.

b) Control of Corneal Thickness:[1,15] It has been seen that when the cornea is exposed to aqueous solutions in vitro or in vivo it has a tendency to swell due to the imbibition of fluid. Because of the presence of a negative imbibition pressure it must be assumed that the cellular layers prevent and control this swelling under normal conditions. Many investigators have now shown this to be true with studies of temperature-reversal and Ouabain inhibition of metabolic activity in the cellular layers. These workers have shown that as temperature decreases the turgescence of the cornea increases and the cellular metabolic activity decreases. With the increase in water content there also occurs an increase in NaCl content although the concentration of the ions does not change. With the return of the temperature to normal there is a decrease in thickness (due to the loss of water) and a loss of Na ions although this is slightly delayed.

When the cellular layers are removed, especially in the case of the endothelium, the hydration of the cornea (and therefore the thickness) increases markedly. Also the return to normal after temperature is once again normal is arrested. The implication has therefore been made by some that the site of the main active transport mechanism controlling hydration-dehydration and

thickness is in the endothelium. Others have shown electric potentials across the epithelial layer and postulated that the main active site was there. At present the consensus is that the active site is in the endothelium and that the large electric potential across the epithelium has nothing to do with the dehydration properties of the cornea.

The nature of the active transport is also rather controversial at present. Four theories have been advanced.[26]

i) An outward primary water pump.

ii) An outward primary ion pump from stroma to aqueous. The ion is probably sodium.

iii) Pinocytosis.

iv) An inward ion pump (sodium) through the endothelial cell into the intercellular space.

The first theory has not been well supported and evidence for it is rather scant. The outward ion pump appears to be well supported. The ion mentioned is sodium and with its active transport out of the cornea, an anion would likely follow. This would produce a hypertonic aqueous with ability to draw water from the cornea. The epithelium, however, has been shown to pump sodium into the stroma and the possibility of antagonistic action between cell layers when both are involved in the control of corneal thickness appears rather unlikely.

Pinocytotic activity has been shown to exist at the endothelial cell layer and can function in both directions. The actual establishment of it as a critical mechanism for dehydration,

however, has not been accomplished.

The inward sodium pump theory has only recently been postulated and evidence to support it is rather scant and difficult to reproduce.

It will be seen therefore that not only the substance transported but also the site of the transport is rather difficult to prove. The control of corneal thickness presently known appears to consist of a balance of the passive movement of water into the stroma due to inherent swelling forces and the active transport of some substance by one of the cellular layers resulting in the loss of water from the stroma.

IV. MATERIALS, METHODS AND PROCEDURES

A. SELECTION OF SUBJECTS

The subjects were adult cats 8 months of age or over, weighing 1.5 to 3.0 kg. and suffering from no systemic or ocular pathology.

B. EQUIPMENT

a) Slit Lamp: The slit lamp used was a Haag-Streit-AG-900. The angle between the light source and the binocular biomicroscope was set at 35° for all measurements. The illumination source is an incandescent lamp that is regulated manually by a variable transformer (from 5 to 7.5 volt range) attached beneath the instrument table.

b) The Pachymeter: The Haag-Streit pachymeter attachment number I, serial number H9000024 for measurements up to 1.2 ml. thickness, was used. This pachymeter is attached to the biomicroscope portion of the Haag-Streit slit lamp and has a slitted bar designed to make constant the angular deviation of the slit light source from the visual axis of the biomicroscope. The 10x eye pieces were used with the pachymeter attachment.

c) Aluminum Clamp: This clamp was designed to hold the enucleated eyes in their transport cages at right angles to the biomicroscope in order to aid in viewing of the corneas.

d) Microscope: The Leitz Laborlux microscope with binocular eye piece tubes S and condenser #601 was found to be an excellent stable instrument expecially when equipped with the heavy metal base to aid in microphotography. The light source is a 6 volt, 15 watt, low

voltage incandescent lamp with a normal maximum load of 2.5 amps. The transformer (REROW) used was set by the factory at 220 volts.

The 10x eye piece was used at all times and the four objective lenses varied in resolving power as follows: 2.5/.07, 10/.30, 40/.65, and the oil immersion lens 100/1.32. The microscope was also equipped with a mechanical stage that was manually adjustable.

e) Camera: The Leitz Orthomat automatic camera attachment was recommended and, therefore, used for the Leitz microscope. This camera is almost completely automatic and comes equipped with the variable object dark/light ratio, automatic ASA setting adjustment, and automatic exposure counter.

f) Incubator: This is a CENCO Gravity Circulation incubator #46068. It is supplied by a 115 volt power source and has a temperature range from 30° - 65°C. Its control sensitivity is plus or minus 1/4°C. and its temperature uniformity at 37°C. is plus or minus 1/4°C. It is based on the principle of gravity air circulation.

g) Hot Air Sterilizer - Model #1420/26K: This model is powered by 115 volt power source and draws 535 watts maximum load. It is based on the principle of super heated air and comes equipped with a mercury type thermometer accurate to plus or minus 2°F. There is also a timer supplied for control of sterilization time.

h) Instruments: A tray was developed to transport the enucleation instruments. The instruments were enucleation scissors, Castroveijo forceps, fixation forceps that were of the locking as well as non-locking variety, the pins and clamps needed to fix the enucleated eyes, and the sealable bottles standard for transport of enucleated

eyes used in corneal transplants. The corneal trephines used to remove the corneal button for staining purposes were acquired from the University Hospital Surgical complex as they were considered to be discards for human useage.

i) Oxygen Tank and Rack: This was merely the standard pressurized oxygen tank used in almost all hospitals in this area.

C. MATERIALS

a)*Lissamine Green: A negatively charged, non-toxic, triphenylmethane dye. Its old colour index number is 737 and its new number is 44090. It is acidic in nature and has been shown to penetrate only cells showing characteristics of irreversible cell damage or death. The penetration has been shown to take place almost immediately after exposure to the dye. The dye particles are apparently bound to the denatured protein within the cytoplasm and nucleus of the cell after the damage has taken place. It is incorporated within the cell as minute particles which become progressively larger and become visible as clumps of a blueish green colour under the light microscope. This dye was selected because of its simplicity of technique, reported accuracy of indication of cellular viability, sensitivity, and apparent lack of interpretive skill necessary to use the stain in a practical manner. The stain in its powder form was dissolved in normal saline in order to reach a dye concentration of 1%.

b) Sterile Normal Saline: This was also used as the basis of the moist

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*Lissamine Green - Esbe Pharmaceutical Supplies, Toronto, Ontario.

atmospheric conditions necessary within the enucleation jars used to store the freshly enucleated eyes.

c)*Eagles Maintenance Medium: A maintenance medium used mainly for tissue culture and virology techniques and has the following components:

i) Amino Acids: These are all of the Levo-variety and consist of the following: Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine, Tyrosine, Cystine, and Glutamine.

ii) Vitamins: Thiamine, Riboflavin, Choline, Pyridoxal, Folic Acid, Inositol, Nicotinamide, and Pantothenate.

iii) Salts: Na Cl, K Cl, $MgCl_2 \cdot 6H_2O$, $CaCl_2$, $NaH_2PO_4 \cdot 2H_2O$, $NaHCO_3$.

iv) Others: Glucose and Phenol red (pH indicator). This maintenance medium contains all the components necessary for tissue culture and cells to continue living in the proper atmosphere.

One oddity of the maintenance medium is that upon contact with oxygen or prolonged exposure to the atmosphere it changes to a purple colour from its natural red. This, however, is no reflection of pH change and appears to have no influence on any tissue culture stored in it.[90]

d) Neosporin Ophthalmic Drops: These were used to prevent bacterial contamination. They were instilled into the maintenance medium and upon the corneal surface after enucleation, prior to incubation and

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*Eagles Maintenance Medium - Department of Virology, University of Alberta Hospital, Provincial Laboratory, Edmonton, Alberta.

at other times to wash the cornea.

e) 95% Oxygen: This was bubbled through the maintenance medium during the incubation period in order to maintain aerobic metabolism in the cornea.

f) Nembutal (Pentobarbital Sodium): 50 mg. per cc. was instilled via intracardiac injection into the blood stream of the cats as a humane and rapid method of sacrificing the animals.

g) Photographic Film: This was the Kodachrome Professonal II (ASA 40).

D. METHODS AND PROCEDURES

a) Calibration of Pachymeter: To obtain a facsimile of the cornea, a glass tube whose outer diameter approximated the anterior corneal diameter and whose inner diameter was ground to approximate the posterior corneal diameter was chosen. A small area of the glass tube was arbitrarily selected and measured using an engineers dial gauge and then filled with liquid paraffin. The paraffin was used to simulate the aqueous humour. An average of six readings with the dial gauge was taken to be an accurate measurement of the thickness of the corneal model selected. The arithmetic mean of the measurement taken by the dial guage was $0.409 \pm .004$ mm.

The corneal model was then measured optically by the pachymeter. The slit light source and the biomicroscope were set at a 35^{o} angle and six readings of the selected area on the corneal model were taken with a mean value of $0.420 \pm .005$ mm. This, therefore, produced an observer error of 0.011 mm. or 2% which is within the maximum allowable error.

b) Determination of Stain Concentration: Concentrations of Lissamine

green from 0.1% to 2% were tested on various corneal endothelial preparations. It was found that the 1% concentration produced the best visibility and a rapid uptake of the dye. At that concentration there was no necessity to wash the corneal surface and produce some additional damage. The Lissamine green was mixed with normal saline on a weekly basis, as it has been found by other investigators that the stain colour seems to deteriorate after a week in its aqueous form.

c) Enucleation Procedure: Following death of the animal the eyes were immediately enucleated under semi-sterile conditions. The upper and lower lids were dissected to the orbital margins and reflected out of the operative field. Using fixation forceps and enucleation scissors the muscles were divided. The optic nerve was then severed and the eye removed. Fixation to the eye cages was by pins through the optic nerve which had been placed through a hole in the base of the cage. The eyes were then inserted into the enucleation jars over gauze soaked in sterile normal saline. Neosporin was dropped over the corneal surface, the jars were sealed and placed within containers generally used throughout North America for transport of enucleated eyes for transplantation.

d) Temperature-Reversal Procedure: Within 20 minutes of enucleation each intact eye was examined by slit lamp and the corneal thickness measured with the pachymeter after the method of Maurice and Giardini.[63] Each cornea was measured six times and the arithmetic mean calculated from these readings.

Following these measurements the intact eyes were refrigerated

in a moist atmosphere at approximately 4°C. for periods of 5, 24 and 48 hours. Following each cooling period the corneas were re-measured with the pachymeter. The normal saline previously placed in the jar was then emptied and replaced with Eagles Maintenance Medium. Neosporin drops were dropped on the surface and each eye in its clamp was replaced within its own jar. The eyes were then incubated for 2 hours at 37°C. During this incubation period, oxygen was bubbled into the maintenance medium through a small nasal oxygen catheter in order that aerobic metabolism would continue. Following the incubation the corneas were again measured with the pachymeter and the arithmetic mean calculated to determine the change in thickness produced by incubation in a nutrient medium.

e) Staining Procedure: Due to the fact that Lissamine green is supplied as a powder it had to be first reconstituted with enough sterile normal saline to produce a 1% solution (1 gram/100 cc.). After the eyes had been removed from their cages in the transport jars a 6 mm. button or disc was trephined from the central corneal region. This method followed the procedure used in taking the corneal button from the donor eye in any transplantation procedure. Because of the sensitivity of the Lissamine green stain we found that separation of the cornea with 2 - 3 mm. of sclera attached, invariably produced a gush of aqueous humor and a consequent displacement of the iris onto the endothelial surface of the cornea. This we found to produce a variable influence upon the integrity of the endothelial cells but generally speaking it was found to produce cell damage in a certain population of the endothelial cells. We, therefore, attempted to

trephine the cornea from the rest of the enucleated eye. The central portion (approx. 3 - 5 mm.) of the cornea has been shown by Maurice and Giardini[63] to be relatively of more constant thickness than any other portion. By trephining this optical or central zone then, one overcomes the problem of dealing with the more peripheral portions of the cornea under the microscope camera.

After the wholemount was placed on the microscope slide it was found that a fairly uncluttered view of the endothelial surface of the cornea was available intact, at least in the central part involving the corneal button, with no radial cuts to interfere with its integrity.

As most other workers had used Lissamine green on a cell suspension the staining technique had to be altered for use with a wholemount specimen. A few drops of the stain were gently placed on the endothelial surface for 20 - 25 minutes. The excess stain was then removed from the endothelial surface by blotting paper without touching it. No washing solution was used as this could possibly have damaged the surface. The slide then was placed under the microscope and examined first under low power, to find the exact geometric center of the button, and then under the next higher power, to observe and photograph the stained cells. The first photograph was always the geometric center of the corneal button. Following this, with the aid of the mechanical stage, eight other photographs were taken of areas immediately surrounding the center (Fig. 1). Each of these areas was photographed regardless of their appearance to insure consistency in the location of the areas of each cornea to be analysed.

f) Analysis of Endothelial Staining: In order to determine the relationship between the number of cells stained on the completely dead endothelial surface and that on the surface of a cornea analysed during storage the endothelium of a cornea had to be killed although left intact. This general cell death was accomplished by heating these corneas to approximately 60°C. for about 10 minutes. The endothelial surface of the cornea was then stained and photography showed that the entire field was covered with stained cells (Fig. 6). These stained cells were counted using a white blood cell counter and it was found that there was an average of 2023 cells per medium power field. After the total number of cells liable to exposure to the stain on one slide was determined as noted above, each cornea that participated in the procedures was then analysed by counting the number of endothelial cells stained in each of the photographs and the mean value calculated.

g) Statistical Analysis: Our experimental results were analysed by two statistical formulae as follows:

where N = number of members of a series

x + y = the measurements of the 2 variables to be correlated

Σ = sum

i) Correlation Coefficient[3]:

formula - $$r_{xy} = \frac{(N\Sigma xy)-(\Sigma x \Sigma y)}{\sqrt{N(\Sigma x^2)-(\Sigma x)^2 \quad N(\Sigma y^2)-(\Sigma y)^2}}$$

This formula was used to discover if a statistical correlation existed between the number of dead cells indicated by staining with Lissamine green and the time in storage following enucleation. It was also used to investigate a possible correlation

between the percentage thickness change that occurred after incubation and the various storage time periods. A correlation between the number of dead cells and the Temperature-reversal effect was also sought.

The statistical significance of the correlation coefficient itself is implied by a value that approaches unity but may be separately calculated when values of r are borderline. The formula used was:

$$T = r_{xy}\sqrt{\frac{N - 2}{(1 - r_{xy}^{2})}}$$

ii) Student's Test for the Difference Between Two Means[3]: This test was used in establishing the significance of the difference between mean changes in thickness during different storage times. This formula is:

$$t = \frac{\overline{x_1} - \overline{x_2}}{\hat{s}}\sqrt{\frac{n_1 n_2}{n_1 + n_2}}$$

where n = number of members of a series

$\overline{x}$ = the mean of that series

$\hat{s}$ = the standard error

V. RESULTS

No Temperature-reversal procedures were attempted on the corneas observed immediately following enucleation because there was no cooling period to produce an increased thickness. The endothelium however was examined microscopically after staining with Lissamine green.

Nine intact eyes were cooled for 5 hours then analysed for thickness change and again 2 hours later for endothelial staining with Lissamine green, as well as for Temperature-reversal effect. The third group of observations on 10 corneas began after 24 hours cooling at 4°C. by observing thickness change that had occurred, followed by Temperature-reversal and endothelial staining observations.

Finally the last group of figures was collected by observing thickness change occuring in 4 corneas after 48 hours of cooling and then after 2 hours incubation. The effect of the Temperature-reversal as well as the cellular staining with Lissamine green was seen at that time.

The following results are based on observations of the changes occuring in the corneas of eyes enucleated from common household cats at four different time periods following enucleation. These time periods were selected in order to give some indication of the changes that occur in a cornea stored for a short time in a moist atmosphere prior to keratoplasty.

The age, weight, and size of the cats used all influenced the size of the cornea and therefore its thickness. The absolute value of corneal thickness therefore does not give an accurate portrayal of the effect of Temperature-reversal in more than one subject. It was therefore decided that the percentage change in corneal thickness was a better indicator

of the effect of cooling than the absolute value. Table II shows the mean increases in thickness of the corneas analysed after refrigeration and Table III shows the decreases in thickness after incubation (also Fig. 2). The percentage decrease in thickness is considered to be a function of the corneal viability. The difference between the mean thickness decrease that occurs in the corneas stored for 7 hours (16.38% $\pm$ 5.77) and that which occurs in the corneas stored for 26 hours (12.56% $\pm$ 3.09) is not significant ($P < .5$). The difference, however, between the mean thickness decrease of corneas stored for 26 hours and those stored for 50 hours (7.37% $\pm$ 1.20) following enucleation is highly significant ($P < .01$). The difference in mean thickness decrease between the corneas stored for 50 hours and those of the corneas stored for 7 hours is also significant ($P < .02$).

While observing the endothelial surface after staining was completed we noted that there were many large areas that were unstained. We then had to decide whether these were unstained cells or areas with no cells present in them. If the corneal lamellae were observed in these areas with distinct clarity it was assumed that no cells were present. If, however, the view of the lamellae was somewhat indistinct this was interpreted as being due to the presence of endothelial cells. This was proven by killing the endothelium by heating while observation was in process. In the areas that had previously been slightly clouded stained cells appeared while in areas that were relatively clear nothing changed.

The sensitivity of the stain was well demonstrated when a fine camel's hair brush was gently passed over the endothelial surface (Fig. 3) and also when a small strand of gauze was dropped on the endothelial

surface just prior to staining (Figs. 4 and 5)

We also found that a number of cells on the endothelial surface had rather different morphology. This appeared to be explained by the observation that as time after death of the cell was extended before the stain was applied a different appearance was noted. If stained immediately following death the appearance in Fig. 7 was noted. If left 24 hours (Fig. 8) or 48 hours (Fig. 9) the appearance changed with the nucleus becoming more consolidated and the cytoplasm/nucleus ratio much increased.

The appearance of the human corneal endothelium examined was much different than that of the cat. We found that for the same amount of time in storage a human cornea would contain far more stained endothelial cells than that seen in the cat.(Figs. 10, 11 and 13). The appearance of a hole present in the endothelial surface is well illustrated in Fig. 12.

It was found during the incubation process that the tissue culture medium above which the enucleated eyes were stored and through which the 95% oxygen was bubbled, appeared to have changed colour from a bright red to a light purple. Others have shown that this change bears no relation to any pH change but appears to be related to the quantity of oxygen or air to which the medium is exposed.[90]

The data obtained via endothelial cell counts was accumulated after the same time intervals after enucleation as the data on the Temperature-reversal effect. A cell count was first made on each cornea of nine subjects immediately following enucleation to provide a baseline for comparison with results obtained after storage. Table IV shows that the mean cell count immediately after enucleation was 13.68 cells per photograph. The area of each photograph was .360 mm.^{2}. The mean count

obtained 7 hours after enucleation was 26.93, 26 hours after enucleation 91.84 and 50 hours after enucleation 99.25. Each value in the table was the mean count of nine photographs of adjacent areas of each cornea. Table IV suggests that an increase in time following enucleation is associated with a rising mean cell count of the corneal surface. Because the photographs are taken to be representative of the entire corneal surface we may then assume that the cell count is increased across the entire cornea as well.

Fig. 14 relating mean cell count to time in storage brought to our attention a very interesting observation. The wide variation of the standard error at 24 hours as compared to the other time coordinates was most unexpected. The possibility that this variation could be due to individual idiosyncracy is very high when regarded in the light of very small standard errors in the other time period calculations.

The difference in mean cell counts of eyes immediately after enucleation and means in eyes stored for 7 hours is highly significant ($P < .01$). This suggests that the differences between mean values obtained immediately following enucleation and those obtained at 26 and 50 hours of cold storage are even more significant ($P < .001$). In other words a significant relationship has been found relating time after enucleation and cell death.

Statistical analysis comparing the staining method using Lissamine green and the Temperature-reversal effect produce the following results:

A correlation coefficient was calculated relating various cell counts to Time Following Enucleation. This figure + .717 was found to be highly significant ($P < .01$). This infers that the cell count is

related to the increase in time of storage following enucleation. The correlation coefficient calculated when the various cell counts were related to the decreasing thickness change was found to be $-.460$ ($P < .02$). Fig. 15 illustrates this correlation graphically.

TABLE II

Percentage Increase in Corneal Thickness Following Refrigeration 4°C.

Cornea	Time (in hrs.) Following Enucleation			
	0	5	24	48
1	-	23.7	53.4	54.0
2	-	18.4	59.7	53.7
3	-	21.0	64.1	66.9
4	-	22.1	64.4	75.9
5	-	14.1	55.5	-
6	-	41.1	49.1	-
7	-	21.1	31.6	-
8	-	18.5	62.6	-
9	-	15.1	46.4	-
10	-	-	47.3	-
Mean	-	21.68%	53.41%	62.62%
S.D.		7.93	10.24	10.79

TABLE III

Percentage Reduction in Corneal Thickness Following Incubation at 37°C.

Cornea	Time (in hrs.) Following Enucleation			
	0	7	26	50
1	-	19.6	15.2	7.5
2	-	13.5	11.9	5.9
3	-	16.6	14.0	7.3
4	-	16.0	7.6	8.8
5	-	9.6	11.5	-
6	-	29.1	9.3	-
7	-	18.6	17.4	-
8	-	12.4	9.6	-
9	-	12.1	15.0	-
10	-	-	14.1	-
Mean	-	16.38%	12.56%	7.37%
S.D.		±5.77	±3.09	±1.20

TABLE IV

Mean Cell Count Following Storage

Cornea	Time (in hrs.) Following Enucleation 0	7	26	50
1	12.6	16.4	94.0	87.2
2	15.8	34.6	134.8	101.7
3	21.5	33.0	38.2	101.8
4	10.0	30.0	176.8	106.3
5	3.4	45.6	113.6	-
6	16.4	19.3	73.4	-
7	7.5	34.8	160.6	-
8	13.7	18.2	66.4	-
9	22.2	10.5	20.1	-
10	-	-	40.5	-
Mean	13.68	26.93	91.84	99.25
S.D.	6.15	8.76	53.6	8.30

See Fig. 14.

VI. DISCUSSION

The procedures in this research were devised in order to find a simple method to measure the viability of a cornea under storage conditions comparable to those used in eye banks. Methods reported by investigators in other centres are too complicated for routine use in this situation.

Early in the experiment, it became obvious that a new staining technique for determining corneal endothelial viability had been developed. The choice of Lissamine green as the supravital stain was based mainly upon work by Holmberg[42] in his excellent paper describing the impressive efficacy of the dye as compared to others. More recent literature written by Kaufman and others advocates the use of Nitro-blue tetrazolium as a simple, straight-forward technique of determining the viability of corneal endothelial cells. Lissamine green, however, requires much less effort than that required in the technique using Nitro-blue tetrazolium and in addition produces excellent morphological representations of the cells involved.

A number of publications have been made in recent years that have produced some confusion as to the interpretation of results arrived at with Nitro-blue tetrazolium. This confusion revolves around the differences in technique prior to incubation of the material with the stain, as well as the differences in opinion regarding the actual significance of a stained cell.

The method by Kaufman[45] et al differs from the method by Pẽna-Carrillo and Polack[69] in that the corneas are quick frozen initially in order to make the endothelial cells permeable. This (in essence) allows the respiratory

enzymes to escape from cells already dead and therefore the cells remain unstained. The second method mentioned does not rely on this factor.

Regardless of the above factors, however, there also appears to be a difficulty involving the determination of the density of the dye and its relation to so called viability. Other workers have shown that the presence of the stain attached to the respiratory enzymes may be found in dead as well as live cells and that the stain should be questioned as a viability indicator.

A. SUPRAVITAL STAINING WITH LISSAMINE GREEN

As the experiment proceeded we became even more convinced after reviewing the literature in a comparative sense that our staining technique and the results thereby obtained were slightly superior to those reported by other investigators.

The Lissamine green portrayed cellular morphology well. There was no color differential involved in the determination of the viability of the cells as might be seen with Eosin-Y or even Nitro-blue tetrazolium.

The simplicity of the technique was quite evident when compared to the incubation procedures necessary with Nitro-blue and its sensitivity appeared to be greater than that seen with Nigrosin and Eosin-Y.

The only stain that appeared to be comparable to Lissamine green was Trypan blue, however, it has been noted as having toxic effects upon the cell that Lissamine green does not.

All these comparisons supported our contention that Lissamine green was the best possible stain available for our purposes. One must remember, however, that a good deal of work must still be done to determine the clinical usage that this stain may be applied to.

B. TEMPERATURE-REVERSAL EFFECT

The procedures involved in this part of the experiment were similar to those reported by Hassard[36] in his monograph on "The Selection of Donor Corneal Tissue". In all cases such corneas examined were measured for thickness three times during the procedure; initially to determine its baseline thickness, secondly to determine its thickness after cooling, and thirdly to determine its thickness after incubation. As there appears to be general agreement in the literature upon the fact that corneas are thicker after longer storage in a moist cool atmosphere than those in a short storage and that this is an effect ascribed to the decrease in metabolic function of the endothelium we will proceed no further in that direction. However, the fact that corneas when stored for longer periods of time and then incubated show less of a thickness decrease, and which layer or layers of the cornea contains the mechanism which produces this thickness decrease becomes a slightly different problem. The results show that as time after death increases the change in thickness after incubation for 2 hours at 37°C. was much less than that encountered at a shorter period of storage.

The correlation coefficient between the cell counts as determined by Lissamine green, and time increase was +.717, and can be seen to be significant ($P < .01$). In other words there appears to be a correlation between an increasing storage time and an increasing cell death as shown by a positive response with Lissamine green. Also there appears to be a correlation of storage time and decreasing amount of change in corneal thickness as time after enucleation increased.

As can be seen in Fig. 14 the standard deviations of values determined at periods immediately following enucleation, 7 hours after

enucleation and 50 hours after enucleation were all rather small and there appeared to be very little scatter in those time periods. However, at the 26 hour time period following enucleation there was an extremely large standard deviation present. This large figure may be due to an inadequate sample size, however, one feels that there is possibly a second explanation, that being the inherent variability that might be present in each of the subject corneas.

Statistically speaking a correlation was evolved between the cell counts established by the Lissamine green technique and the Temperature-reversal effect, in particular the decrease following incubation. This correlation value was -.460 and was found to be significant ($P < .05$). In other words there appears to be a relationship between the increasing cell death as indicated by cells stained with Lissamine green and the lack of thickness decrease as time after storage becomes longer. The present evidence merely substantiates that produced by other workers, [12, 13, 14, 33, 55, 56, 57, 64, 66, 75,] however, in a slightly different manner. One could postulate then that the endothelial integrity is the main influence upon the hydration-dehydration mechanism of the cornea.

As can be seen in Fig. 2 the decreasing change in thickness that occurred following incubation as time after enucleation increased, was rather uniform and very unlike Fig. 14 in which the cell death as indicated by cells stained with Lissamine green was a slightly uneven rise. This very difference in the graphical representation of the experimental function displays the answer to the problem as originally set at the beginning of the experiment Because of the fact that there is a uniform and not a precipitous or otherwise acute change in the thickness graph

there cannot be a distinct point in time (at least up to 48 hours) that one may say the cornea by this method is either viable or not viable at that point. There seems to be, however, very good evidence that the Lissamine green technique produces just such a curve. It shows that apparently a good percentage of the cells observed on the endothelial surface die in the first 24 hours, and then the rate of death decreases over the next 24 hours. After this, however, the form of the graphical illustration can only be left to speculation and future experimental work.

It has been well documented that human corneas stored for a period of 48 hours are considered very poor for donor purposes. After staining the human corneas received by our laboratory after that period of storage or less it was not difficult to understand why. Masses of cells were stained and almost the entire cornea was covered by them.

When observing the cat corneas stored for 48 hours it was found that approximately 5% of the cells only were stained after 48 hours storage. This was a marked difference when compared to the human corneas.

It appears that endothelium present on the cat's cornea is much more resistant to damage than that present on human endothelium. The comparison of a cat and human corneas would therefore be invalid if this were not considered.

SUMMARY AND CONCLUSION

The study undertaken in this case was made in order to establish a method that was simple, direct and accurate in measuring the viability of the cornea. It was decided to compare a method evolved in this laboratory using Lissamine green as a supravital stain and the Temperature-reversal effect following the methods of Hassard in 1964.

It was shown that the Temperature-reversal effect produced percentage changes in thickness of a uniform nature even over irregular time periods and therefore produced a situation in which no one could tell at any particular time whether a cornea was completely viable or not. The method using Lissamine green as a supravital technique, however, displayed a very accurate and sensitive indication of cellular death. From this was developed a graphical relationship upon which was plotted points relating cell death and time following storage. This graph if extended to include more time and if expanded to include human donor material may indicate a particular point at which viability gives way to non-viability.

It appeared that the Lissamine green staining results as described by Holmberg and ourselves were much superior to those produced by Nitro-blue tetrazolium as a staining technique. We could, therefore, conclude:

1. That a supravital staining technique for corneal endothelium involving the use of Lissamine green is much superior to any staining method that has been reported in the literature recently or in the past.
2. That the above staining technique is a simple, direct and highly

sensitive method of indicating cellular viability even in a one cell layer thick membrane such as the corneal endothelium.

3. That the Temperature-reversal effect in the method used by our laboratory is inadequate as a measure of viability or non-viability of the cornea.

4. That a long-term storage study must be undertaken following these experiments in order to find the projected point at which all cells on the endothelial surface would be dead and thus stained by Lissamine green.

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Figure 1. The pattern followed in taking photographs of all corneal endothelial surfaces.

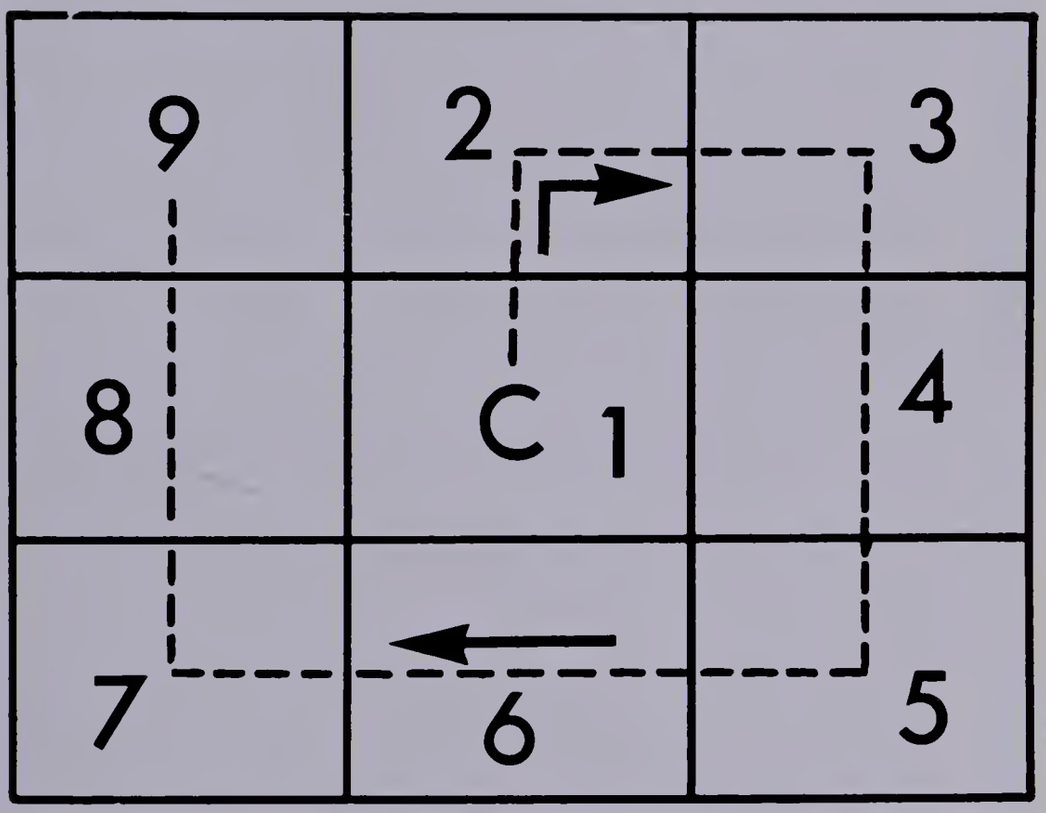

9
2
3
8
C
1
4
7
6
5

Figure 2. Graph I - The relationship found between mean percentage decrease in corneal thickness and various storage times.
Dotted lines represent one standard deviation.

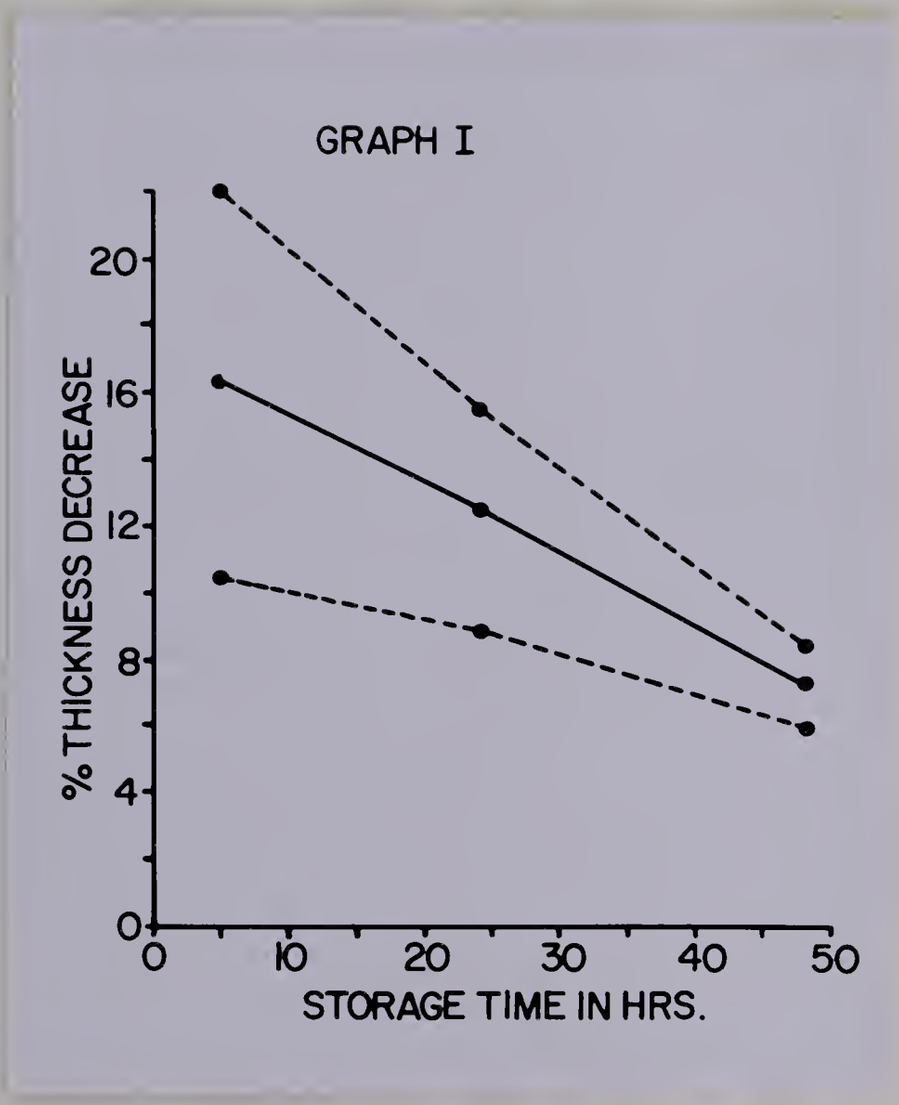

GRAPH I
% THICKNESS DECREASE
0
4
8
12
16
20
0
10
20
30
40
50
STORAGE TIME IN HRS.

Figure 3. The appearance of corneal endothelium of a cat following a light stroke over its surface with a camel hair brush (X100)

Figure 4. Corneal endothelium of a cat after a strand of gauze was dropped on its surface and then stained (X100).

Figure 5. Corneal endothelium of a cat after a strand of gauze was dropped on its surface and then stained (X400).

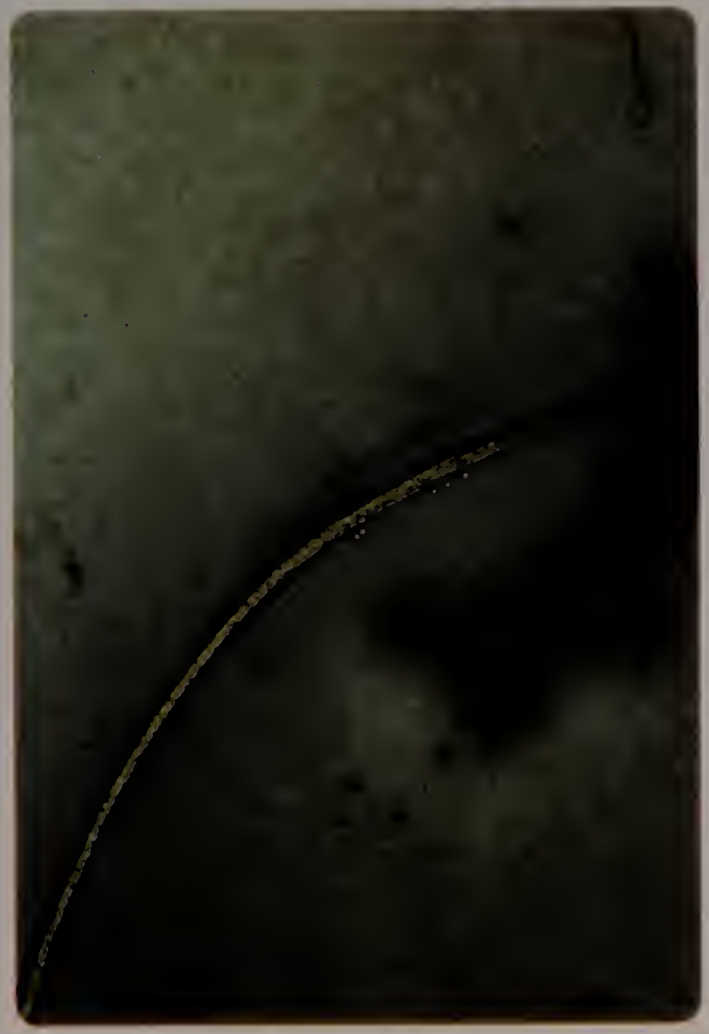


Figure 6. The appearance of corneal endothelium of a cat after heating acutely at 60°C. (X100).

Figure 7. The appearance of the corneal endothelium of a cat stained immediately following the death of cells. The nucleus is large with a small amount of cytoplasm around it (X400).

Figure 8. The appearance of the corneal endothelium of a cat stained approximately twenty-four hours after the death of the cells. The nucleus is smaller, darker and more consolidated. There is more cytoplasm around the nucleus and it appears granular (X400.)

Figure 9. The appearance of the corneal endothelium of a cat stained approximately forty-eight hours after the death of the cells. The nucleus is quite consolidated and small with a large amount of cytoplasm surrounding it that has lost its granularity (X400).

Figure 10. Human corneal endothelium stained approximately twenty-four hours after enucleation. Shows early vacuolation of some cellular elements (X100).

Figure 11. Human corneal endothelium stained approximately twelve hours after enucleation. Little if any vacuolation present (X100).

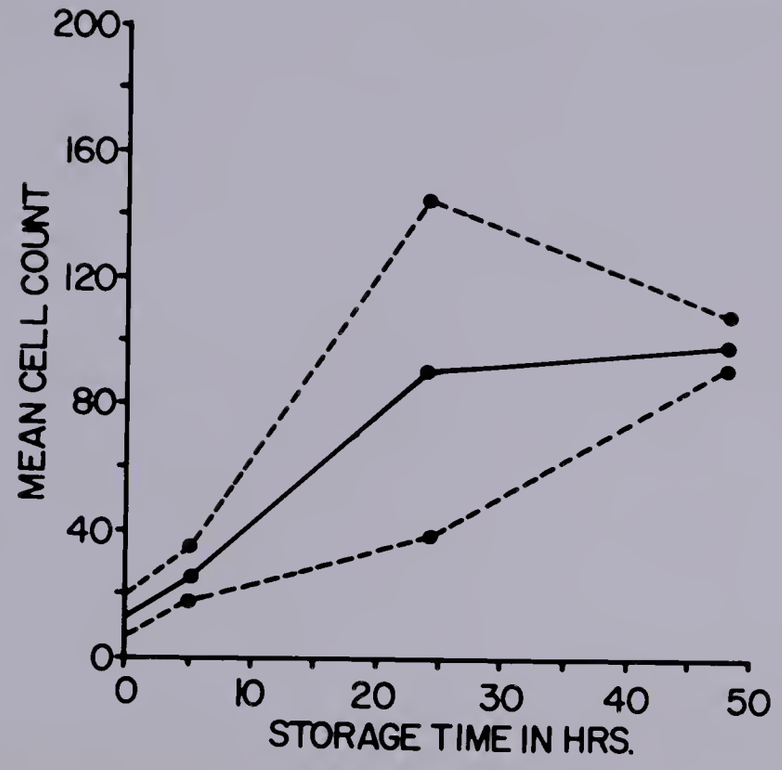
Figure 12. Human corneal endothelial surface showing a small tear (X400).

Figure 13. Human corneal endothelium stained approximately twelve hours after enucleation. Outlines of dead endothelial cells are well portrayed (X400).

Figure 14. Graph II - The relationship found between mean cell count and various storage times. Dotted lines represent one standard deviation.

Figure 15. Graph III - The relationship found between mean cell count and mean percentage decrease in corneal thickness.

GRAPH II



GRAPH III